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The *Xenopus* Suc1/Cks Protein Promotes the Phosphorylation of G_2/M Regulators*

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The entry into mitosis is controlled by Cdc2/cyclin B, also known as maturation or M-phase promoting factor (MPF). In *Xenopus* egg extracts, the inhibitory phosphorylations of Cdc2 on Tyr-15 and Thr-14 are controlled by the phosphatase Cdc25 and the kinases Myt1 and Wee1. At mitosis, Cdc25 is activated and Myt1 and Wee1 are inactivated through phosphorylation by multiple kinases, including Cdc2 itself. The Cdc2-associated Suc1/Cks1 protein (p9) is also essential for entry of egg extracts into mitosis, but the molecular basis of this requirement has been unknown. We find that p9 strongly stimulates the regulatory phosphorylations of Cdc25, Myt1, and Wee1 that are carried out by the Cdc2/cyclin B complex. Overexpression of the prolyl isomerase Pin1, which binds to the hyperphosphorylated forms of Cdc25, Myt1, and Wee1 found at M-phase, is known to block the initiation of mitosis in egg extracts. We have observed that Pin1 specifically antagonizes the stimulatory effect of p9 on phosphorylation of Cdc25 by Cdc2/cyclin B. This observation could explain why overexpression of Pin1 inhibits mitotic initiation. These findings suggest that p9 promotes the entry into mitosis by facilitating phosphorylation of the key upstream regulators of Cdc2.

The entry into mitosis in eukaryotic cells is controlled by maturation or M-phase promoting factor (MPF)[1] (1). In *Xenopus* egg extracts, MPF is a trimeric complex consisting of the protein kinase Cdc2, a B-type cyclin, and a small 9-kDa subunit called Xe-p9 (p9), a homolog of the Suc1/Cks protein (2). MPF acts by carrying out key regulatory phosphorylations on various substrates that are involved in mitotic processes such as nuclear disassembly and chromosome transmission. In addition, MPF participates in regulatory circuits that control its "autocatalytic" activation at the G_2/M transition (3, 4) and its inactivation at the conclusion of M-phase, respectively (1).

During interphase, Cdc2 is kept inactive because of inhibitory phosphorylations on its Tyr-15 and Thr-14 residues. These modifications are catalyzed by the kinases Wee1 and Myt1 (1). The dual-specificity phosphatase Cdc25 removes both inhibitory phosphates when the conditions are appropriate for mitosis, thereby activating Cdc2 (1). Cdc25, Myt1, and Wee1 are all highly regulated during the cell cycle (5–10). For example, these enzymes become highly phosphorylated at mitosis and concomitantly undergo large changes in their catalytic activities. In particular, the kinases Cdc2/cyclin B and Plx1 collaborate to carry out the stimulatory phosphorylations of Cdc25 (3–5, 11). Conversely, the Cdc2-specific kinase activities of both Myt1 and Wee1 are strongly down-regulated by phosphorylation at mitosis (7–10).

The Suc1/Cks protein is also essential for the proper regulation of Cdc2 in various species (2, 12–15). In *Xenopus* egg extracts, p9 is required for both entry into and exit from mitosis (2). The role of p9 in mitotic exit is now understood in some detail. In particular, p9 is necessary for the destruction of cyclin B and the phosphorylation of various proteins associated with ubiquitin-mediated proteolysis (*e.g.* Cdc27 and BIME) (16). In the case of Cdc27, p9 was shown to strongly enhance the ability of Cdc2/cyclin B to phosphorylate recombinant Cdc27 without affecting the intrinsic catalytic activity of Cdc2, indicating that p9 facilitates substrate recognition (16, 17). Significantly, immunodepletion of p9 also prevents the entry of *Xenopus* interphase egg extracts into M-phase, but the molecular basis of this defect has not been resolved. In the absence of p9, the Cdc2/cyclin B complex accumulates in its inactive form that is phosphorylated on Tyr-15 and Thr-14, suggesting that p9 in some manner regulates the dephosphorylation of these residues.

The Pin1 protein has recently emerged as another potential regulator of mitotic progression (18–22). The human Pin1 protein was identified in a yeast two-hybrid assay by virtue of its ability to interact with the mitotic regulatory kinase NIMA (18). Pin1 is a nuclear protein that has two structural domains: an N-terminal WW domain that is involved in protein-protein interactions and a C-terminal prolyl-isomerase domain. Depletion of Pin1 from HeLa cells results in a mitotic arrest, whereas overexpression of Pin1 in HeLa cells causes a G_2 arrest (18). Similarly, the addition of excess recombinant human His6-Pin1 protein to cycling *Xenopus* egg extracts can block the entry of these extracts into mitosis (20, 21). These studies indicated that Pin1 can impinge upon the Cdc2/cyclin B-dependent pathway of mitotic regulation.

In this report, we have examined the requirement for p9 in regulating the G_2/M transition. We find that p9 significantly stimulates the ability of a Cdc2/cyclin B complex to phosphorylate the mitotic regulators Cdc25, Myt1, and Wee1. This observation suggests that p9 plays an indispensable role in the "autocatalytic" activation of MPF at the G_2/M transition. In the course of these studies, we also observed that excess Pin1 protein can antagonize the stimulatory effect of p9 on the phosphorylation of the regulators of Cdc2, suggesting that Pin1 may likewise participate in the activation of Cdc2.

EXPERIMENTAL PROCEDURES

Analysis of Xenopus Cdc25, Myt1, and Wee1 Proteins—His6-Cdc25 and His6-Myt1 proteins were purified from baculovirus-infected Sf9 cells as described (10, 11). Phosphorylation of these proteins was analyzed using 8% SDS gel electrophoresis. ^{35}S-Labeled *Xenopus* Wee1 was synthesized using pET3a-XeWee1 as the template (7) and the TNT *in vitro* transcription/translation system (Promega Corp.) in the presence

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[1] The abbreviation used is: MPF, M-phase promoting factor.

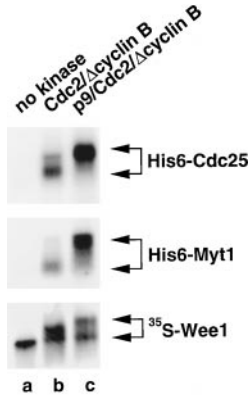

FIG. 1. **p9 strongly enhances phosphorylation of the mitotic regulators Cdc25, Myt1, and Wee1 by Cdc2/cyclin B.** Recombinant His6-Cdc25 (*top panel*), His6-Myt1 (*middle panel*), and ^{35}S-Wee1 (*bottom panel*) were incubated in 30 μl of kinase buffer (5 mM Tris-HCl at pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mg/ml ovalbumin, 1 μM okadaic acid, and 100 μM ATP) without a Cdk complex (*lane a*), with Cdc2/Δcyclin B (*lane b*), or with p9/Cdc2/Δcyclin B (*lane c*). Recombinant Cdc2/Δcyclin B and p9/Cdc2/Δcyclin B were prepared as described (16) and used at a final concentration of ~50 nM (Δcyclin B is a histidine-tagged form of the human cyclin B1 protein lacking the N-terminal 87 amino acids which contain the cyclin destruction box). Reactions with His6-Cdc25 and His6-Myt1 also contained 5 μCi [γ-^{32}P]ATP. His6-Cdc25 and His6-Myt1 were used at a final concentration of ~300 nM. All kinase reactions were incubated at 22 °C for 15 min and then subjected to SDS-polyacrylamide gel electrophoresis and analyzed with a PhosphorImager (Molecular Dynamics). All experiments were performed a minimum of three times.

of [^{35}S]Translabel (ICN Biomedicals, Inc., Irvine, CA). Analysis of the phosphorylation products was performed by SDS-gel electrophoresis with a 10% polyacrylamide gel containing 0.357% piperazine diacrylamide (Bio-Rad) instead of 0.267% bisacrylamide.

Production of Recombinant Pin1 Proteins—Wild-type His6-Pin1 was expressed and purified from *Escherichia coli* BL21(DE3)pLysS cells using the pET28a-His6-Pin1 construct (18) as described (19) except that the protein was induced at an optical density of 0.5 (600 nm). A plasmid encoding the mutant His6-Pin1-W34A protein was constructed with the QuikChange™ Site-directed Mutagenesis kit (Stratagene), using pET28a-His6-Pin1 as the template (18) and two oligonucleotides with the following sequences: 5′-CACATCACTAACGCTAGCCAGGCGGAGCGGCCCAGCG-3′ and 5′-CGCTGGGCCGCTCCGCCTGGCTAGCGTTAGTGATGTG-3′. This procedure resulted in the conversion of the Trp-34 to Ala (^{100}TGG^{103} to ^{100}GCG^{103}) as well as the insertion of a silent mutation in the coding region of His6-Pin1 (^{91}GCC^{93} to ^{91}GCT^{93}) that allowed for the identification of the mutant clone by restriction analysis with *Nhe*I and *Eco*RV. These mutations were further confirmed by sequencing. The mutant His6-Pin1-W34A protein was then expressed and purified as described above for the wild-type protein.

RESULTS AND DISCUSSION

To assess the role that p9 plays in mitotic entry, we examined whether p9 would affect the Cdc2-dependent phosphorylation of various regulators of the G_2/M transition. For this purpose, we incubated recombinant *Xenopus* Cdc25, Myt1, and Wee1 with a dimeric Cdc2/Δcyclin B complex or trimeric p9/Cdc2/Δcyclin B complex. For Cdc25 and Myt1, phosphorylation was assessed by monitoring ^{32}P incorporation and mobility during gel electrophoresis. As shown in Fig. 1 (*top* and *middle panels*), p9 strongly stimulated the incorporation of radioactive phosphate into both His6-Cdc25 and His6-Myt1. Furthermore, treatment with p9/Cdc2/Δcyclin B, but not Cdc2/Δcyclin B, resulted in a large decrease in the electrophoretic mobilities of both Cdc25 and Myt1, which is characteristic of the mitotic forms of these proteins. The presence of p9 in the Cdc2/Δcyclin B complex consistently resulted in a 4- to 5-fold increase in the appearance of the hyperphosphorylated forms of Cdc25 and Myt1. Because *Xenopus* Wee1 possesses substantial autophosphorylation activity and undergoes less extensive modification at mitosis, we assessed its phosphorylation by monitoring the shifting of ^{35}S-Wee1 in SDS gels under modified electrophoretic conditions (see "Experimental Procedures"). We observed that p9 also significantly increased the phosphorylation of ^{35}S-Wee1 by Cdc2/Δcyclin B, but to a lesser degree than for Cdc25 and Myt1 (Fig. 1, *bottom panel*).

The prolyl isomerase Pin1 binds to the phosphorylated forms of Cdc25, Myt1, and Wee1, as well as other mitotic phosphoproteins near the beginning of M-phase (18–22). Overexpression of Pin1 in human cells (18) and *Xenopus* egg extracts inhibits the entry into mitosis (20, 21). Depletion of Pin1 from human cells by expression of an antisense construct and deletion of the gene encoding the budding yeast Pin1 homologue Ess1 both result in a defect in the progression through mitosis (18). Although the precise physiological role of Pin1 has not been established, these data collectively suggest that Pin1, like p9, plays some role both in the entry into and progression through mitosis.

We asked whether Pin1 might affect the ability of p9 to stimulate substrate recognition by Cdc2/Δcyclin B. Recombinant His6-Pin1 (100 μg/ml) completely abolished the stimulatory effect of p9 on the Cdc2-catalyzed phosphorylation of His6-Cdc25 and His6-Myt1 (Fig. 2*A*). In dose-response studies with His6-Cdc25 as the substrate, we observed that half-maximal inhibition of the p9-stimulated phosphorylation of Cdc25 occurred at ~50 μg/ml His6-Pin1 (Fig. 2*B*). Previously, the endogenous concentration of Pin1 in *Xenopus* egg extracts was estimated to be 0.5 μM or 9 μg/ml (20). Furthermore, recombinant Pin1 at a final concentration of 10 μM (180 μg/ml) completely blocked the entry of egg extracts into mitosis (20). Thus, the concentration of His6-Pin1 that inhibits the p9-dependent phosphorylation of Cdc25 by Cdc2/Δcyclin B is within the range previously established to block mitosis in egg extracts.

In control experiments, we examined whether His6-Pin1 would affect the catalytic activity of p9/Cdc2/Δcyclin B toward the generic substrate histone H1. As shown in Fig. 2*C*, up to 100 μg/ml His6-Pin1 had no effect on the ability of p9/Cdc2/Δcyclin B to phosphorylate histone H1. As reported previously (16), Cdc2/Δcyclin B and p9/Cdc2/Δcyclin B phosphorylated histone H1 with equal efficiency (Fig. 2*C*, compare *lanes a* and *b*). The Cdc25 protein can also be phosphorylated and regulated by the Polo-like kinases (Plx1 in *Xenopus*) (11). However, His6-Pin1 (100 μg/ml) had no inhibitory effect on the ability of Plx1 to phosphorylate Cdc25 *in vitro* (data not shown). Finally, we examined the possibility that His6-Pin1 might disrupt the binding of p9 to Cdc2/Δcyclin B because p9 does possess a proline-containing region that has been implicated in controlling the binding of Suc1/Cks proteins to cyclin-dependent kinases (23–25). By immunoblotting with anti-p9 antibodies (2), we observed that His6-Pin1 (100 μg/ml) did not affect the binding of p9 to Cdc2/Δcyclin B (data not shown).

Pin1 consists of a carboxyl-terminal prolyl isomerase domain and an amino-terminal WW domain that binds to peptides containing phosphoserine or phosphothreonine (22). Its WW domain is critical for the association of Pin1 with mitotic phosphoproteins such as Cdc25. To ask whether the WW domain of Pin1 is necessary for its ability to antagonize p9, we prepared the mutant His6-Pin1-W34A in which the function of the WW domain was abolished by mutagenesis of Trp-34 to Ala. As shown in Fig. 3, the mutant His6-Pin1-W34A, in contrast to wild-type His6-Pin1, was not able to inhibit the p9-stimulated hyperphosphorylation of Cdc25. In contrast, a form of Pin1 with a mutation in the prolyl isomerase domain (Pin1-H59A) (19–20) could still inhibit the stimulatory effect of p9 on the phosphorylation of Cdc25 (data not shown). Thus, the WW

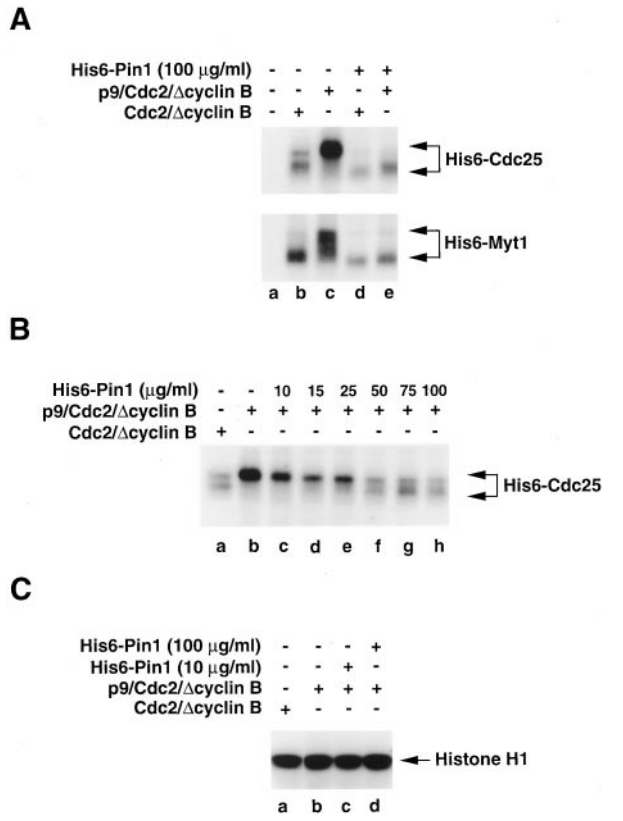

FIG. 2. **The Pin1 protein inhibits the p9-stimulated phosphorylation of substrates by Cdc2/Δcyclin B.** *A*, His6-Cdc25 (*top panel*) and His6-Myt1 (*bottom panel*) were incubated in kinase buffer containing [γ-^{32}P]ATP with no further addition (*lane a*), Cdc2/Δcyclin B (*lanes b* and *d*), or p9/Cdc2/Δcyclin B (*lanes c* and *e*) in the absence (*lanes a–c*) or presence of 100 μg/ml of His6-Pin1 (*lanes d* and *e*). *B*, dose-response experiment for the inhibitory effect of His6-Pin1 on the phosphorylation of His6-Cdc25. His6-Cdc25 was incubated in the presence of [γ-^{32}P]ATP with Cdc2/Δcyclin B (*lane a*) or p9/Cdc2/Δcyclin B (*lanes b–h*) and 10–100 μg/ml His6-Pin1 as indicated (*lanes c–h*). *C*, His6-Pin1 does not affect the histone H1 kinase activity of p9/Cdc2/Δcyclin B. Cdc2/Δcyclin B (*lane a*) and p9/Cdc2/Δcyclin B (*lanes b–d*) were incubated in the presence of [γ-^{32}P]ATP and histone H1 (0.1 mg/ml) either without His6-Pin1 (*lanes a* and *b*) or with either 10 μg/ml His6-Pin1 (*lane c*) or 100 μg/ml His6-Pin1 (*lane d*). The concentrations of His6-Cdc25, His6-Myt1, Cdc2/Δcyclin B, and p9/Cdc2/Δcyclin B were the same as in Fig. 1. All experiments were performed a minimum of three times.

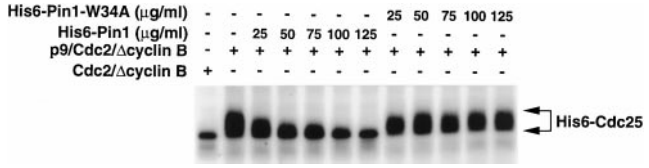

FIG. 3. **The inhibitory effect of His6-Pin1 on the phosphorylation of His6-Cdc25 by p9/Cdc2/Δcyclin B is mediated by the WW domain of Pin1.** His6-Cdc25 was incubated with Cdc2/Δcyclin B or p9/Cdc2/Δcyclin B as described in Figs. 1 and 2 except that 25–125 μg/ml either wild-type His6-Pin1 or the mutant His6-Pin1-W34A was included as indicated. All experiments were performed a minimum of three times.

domain of Pin1 is crucial for its ability to impair the phosphorylation of Cdc25.

In summary, one finding of this report is that p9 strongly enhances the ability of Cdc2 to phosphorylate its key upstream regulators (*e.g.* Cdc25, Myt1, and Wee1) (Fig. 4). Mitotic hyperphosphorylation results in up-regulation of Cdc25 (3–6) and down-regulation of Wee1 (7–9) and Myt1 (10), respectively. Cdc2/cyclin B appears to play a crucial role in these mitotic hyperphosphorylations. Although Plx1 and possibly other kinases participate in this process, it is not fully clear whether Plx1 acts upstream or downstream of Cdc2. Taken together, our results strongly suggest that the arrest of p9-depleted *Xenopus* egg extracts in interphase can be attributed at least in part to a failure in the Cdc2-dependent hyperphosphorylation of Cdc25, Myt1, and Wee1. Consequently, Cdc25 could not be activated and both Myt1 and Wee1 could not be inactivated in the absence of p9. Thus, p9 should be regarded as an essential component of MPF. Conceivably, p9 could play an additional role(s) in regulating the G_2/M transition, such as affecting the ability of Cdc25, Myt1, and Wee1 to modify Cdc2, but we have not been able to detect any effect of p9 on the catalytic functions of Cdc25, Myt1, and Wee1 (data not shown).

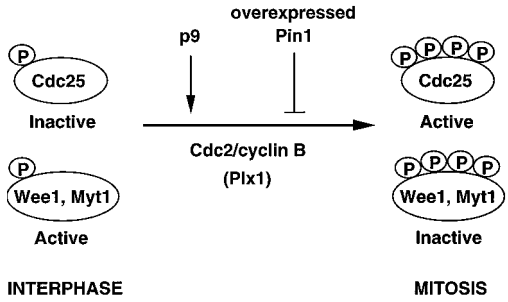

FIG. 4. **Model summarizing the roles of p9 and Pin1 in the mitotic phosphorylation of the Cdc25, Myt1, and Wee1 proteins.** (Note that the exact number of phosphate groups on Cdc25, Myt1, and Wee1 at interphase and mitosis is not known.)

In structural studies, the Suc1/Cks protein has been shown to contain a potential phosphate-binding pocket (25). In principle, this pocket could allow p9 to dock onto phosphoproteins such as Cdc25, Myt1, and Wee1. In this event, p9 could stimulate the further phosphorylation of these proteins by Cdc2/cyclin B through its ability to increase the local concentration of Cdc2 in relation to its substrates. The action of p9 as a substrate-docking factor could help to explain the kinetics of mitotic entry in *Xenopus* egg extracts. In this system, the activation of Cdc2 by cyclin B requires a critical threshold concentration of cyclin B and involves a substantial lag period between the binding of cyclin B to Cdc2 and the ensuing activation of this complex (26).

Another conclusion of this study is that Pin1 can disrupt the stimulatory action of p9 through the ability of its WW domain to bind to the same regulators (*e.g.* Cdc25, Myt1, and Wee1) that are also substrates of p9/Cdc2/cyclin B (Fig. 4). The ability of Pin1 to block the activation of Cdc25 and inactivation of Wee1 and Myt1 could explain why overexpression of Pin1 blocks the entry into mitosis in *Xenopus* egg extracts and human cells. There are conflicting reports about whether Pin1 can directly inhibit the phosphatase activity of Cdc25 (20, 21), but we have not been able to observe an inhibitory effect of human Pin1 on the catalytic activity of *Xenopus* Cdc25 (data not shown). Thus, although Pin1 may be able to inhibit the catalytic function of Cdc25 under certain assay conditions, we suggest that overexpression of Pin1 blocks mitotic entry by compromising phosphorylation of the upstream regulators of Cdc2. These findings raise the possibility that p9 and Pin1 may normally play antagonistic roles in controlling the G_2/M transition. However, at this time, it is not known whether inhibition of mitotic entry by Pin1 could reflect a physiological function of Pin1 or is instead a consequence of its overexpression. Further studies will be required to resolve conclusively the physiological role of Pin1 in cell cycle control.

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